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TI ***Protein*** ***disulfide*** ***isomerase*** . A multifunctional protein resident in the lumen of the endoplasmic reticulum.
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Minireview

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Protein Disulfide Isomerase

A MULTIFUNCTIONAL PROTEIN RESIDENT IN THE LUMEN OF THE ENDOPLASMIC RETICULUM*

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The endoplasmic reticulum (ER)[1] serves as a key junction between the site of initiation of translation of membrane and secretory proteins in the cytoplasm and the routing of these proteins through the endomembrane system. However, it is not merely a junction, for in addition to components associated with the routing of nascent polypeptide chains through the membrane of the ER, such as the signal recognition particle receptor and the signal peptidase, the endoplasmic reticulum contains a host of proteins involved in co- and post-translational modifications of these newly synthesized polypeptides. One subset of this group of proteins resides in the lumen of the endoplasmic reticulum. The most abundant members of this subset are the glucose-regulated proteins, GRP-78 (now more commonly known as BiP), GRP-94, and protein disulfide isomerase, PDI (EC 5.3.4.1). This minireview will focus on PDI.

PDI was so named because it catalyzes the *in vitro* isomerization of intramolecular disulfide bridges. It exhibits a broad specificity and has been used to catalyze the *in vitro* folding of a variety of proteins. Anfinsen and co-workers (1) first isolated the enzyme from liver almost 30 years ago, and it is now clear that the enzyme is present in many organs or tissues (2) and that its primary structure is highly conserved between species. PDI is an abundant protein (0.4% of total cellular protein (3)), and its concentration in the lumen of the ER may approach millimolar levels. Several reviews have appeared recently detailing various aspects of PDI (4–6), and therefore we will focus on the novel properties of PDI that have been the subject of recent papers.

PDI Is a Multifunctional Protein

PDI is also the β-subunit of prolyl hydroxylase (EC 1.14.11.2 (7, 8)). Prolyl hydroxylase catalyzes the post-translational hydroxylation of peptidyl proline residues within pro-α chains of procollagen. The nascent procollagen peptide, α-ketoglutarate, and molecular O_2 are substrates for the reaction, and ascorbic acid and Fe^{2+} are required cofactors (for review see Ref. 9).

Prolyl hydroxylase is a heterotetramer composed of two α- and two β-subunits. The β-subunit (PDI) is found in greater abundance than the α-subunit and also exists alone as a homodimer. The site of binding of the pro-α chains of procollagen is the α-subunit of the enzyme. Because prolyl hydroxylase in *Chlamydomonas reinhardtii* does not contain a β-subunit (10), it is possible that the β-subunit may not play any direct role in the catalytic mechanism of prolyl hydroxylation. In this context, it is of interest that Wells *et al.* (11) have demonstrated that the β-subunit contains dehydroascorbate reductase activity. Since ascorbic acid is a cofactor in the hydroxylation reaction, it may be that the β-subunit (PDI) merely functions to generate one of the required cofactors. It will be interesting to determine if PDI plays a similar role in relation to other enzymes requiring ascorbic acid.

PDI has several other unusual properties. Cheng *et al.* (12) identified by affinity labeling a 3,3′,5-triiodothyronine binding protein (THBP) located in the lumen of the ER and the nuclear envelope that was later shown by sequence analysis to be PDI (13). No data relating the thyroid hormone binding affinity of PDI to any physiological function have been presented; it has been suggested that this activity may relate to effects of thyroxine other than its role in the regulation of transcription (13).

We have demonstrated that PDI has the ability to be specifically covalently labeled by simple tri- or tetrapeptides containing a photoreactive benzoylazido group. Because initially the peptide binding activity of PDI had been identified by using a tripeptide that served as an acceptor for *N*-glycosylation (14), we speculated that this peptide binding activity was associated with oligosaccharyl transferase activity. However, recent evidence suggests that PDI is not required for the transfer of oligosaccharide chains from oligosaccharyl pyrophosphoryldolichol to either nascent polypeptide chains (15) or to acceptor peptides (16). In fact, the peptide binding function of PDI is not limited to peptides containing the *N*-glycosylation site acceptor sequence and the only reason for the apparent preference for glycosylatable peptides was that as a result of their glycosylation they became concentrated in the lumen (17). Although the biological function of the peptide binding activity is unknown, the location of PDI in the lumen of the ER suggests that peptide binding reflects involvement of this molecule in protein folding. Another protein in the lumen of the ER, BiP, also binds proteins and peptides. Protein binding by BiP is abolished in the presence of ATP (18). The effect of ATP on protein binding by BiP appears to be due to an ATPase-dependent mechanism for dissociation of peptides from BiP (19). Our preliminary results[2] suggest that the interaction of PDI with peptides does not exhibit a similar ATPase-dependent release mechanism.

If the peptide binding function of PDI is associated with its activity as a disulfide isomerase it would be expected to exhibit two characteristics. First, the binding should be enhanced for peptides containing cysteine and, second, binding should lack specificity with respect to other amino acid residues, given the variability of sequence in the regions adjacent to disulfide bonds and the diversity of proteins that can serve as substrates. Morjana and Gilbert (20) have recently shown that peptides of various lengths and sequence all acted as competitive inhibitors of PDI activity. Increasing the length of the peptide decreased the K_i, suggesting that PDI preferentially binds longer peptides as substrates and that a large portion of the peptide backbone interacts with PDI. The findings that 1) Cys-containing peptides bind 4–8-fold tighter than peptides of the same length lacking Cys and 2) performic acid oxidation of Cys-containing peptides increased the K_i by 5-fold both indicate some specificity for Cys residues. No other correlation of inhibitory activity was found with charge, hydrophobicity, or sequence of the peptide, suggesting that PDI principally recognizes only the peptide backbone and recognition of the R groups of the individual amino acid residues is limited to the Cys side chain. This broad substrate specificity for the polypeptide backbone, while unusual, would appear to be required for an enzyme of this type and also may explain why PDI may act as a subunit in other enzyme complexes in which recognition of the nascent polypeptide chain is required.

Further support for the idea that PDI binds polypeptides comes from a recent comparison of the effects of synthetic substrates on prolyl hydroxylase. The ability of polyproline to inhibit

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[1] The abbreviations used are: ER, endoplasmic reticulum; PDI, protein disulfide isomerase; THBP, 3,3′,5-triiodothyronine binding protein; PI, phosphatidylinositol.

[2] R. Noiva and W. J. Lennarz, unpublished studies.

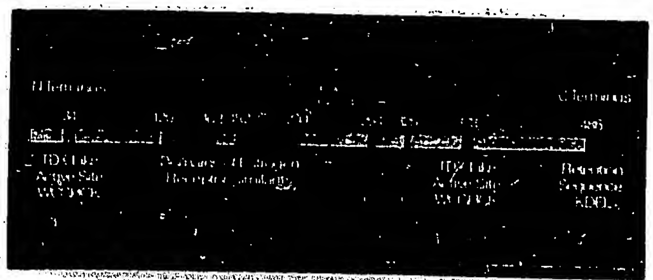

FIG. 1. Diagram of the primary structure of PDI showing the putative active sites and other key features discussed in the text. *TDX*, thioredoxin.

chicken prolyl hydroxylase increased with increased length of the peptide substrate, indicating the longer polypeptide was a better substrate. However, no such chain length effect was noted when the prolyl hydroxylase from *C. reinhardtii* was used (21). Because, as noted above, prolyl hydroxylase isolated from *C. reinhardtii* does not contain a β-subunit (PDI) (10), whereas chicken prolyl hydroxylase does, it seems possible that the PDI subunit, in addition to functioning as dehydroascorbate reductase, enhances recognition of the polypeptide substrate.

Purification of other microsomal enzymatic activities has also yielded preparations containing PDI. Recently this protein was found to be a component of a complex that catalyzes the transfer of triglyceride and cholesteryl ester between membranes (22). The role of PDI in this complex is unknown. PDI has also been suggested to have type I iodothyronine 5-monodeiodinase activity (23, 24). Because of the earlier identification of the thyroxine binding activity of PDI (13), this initially was an attractive hypothesis. However, recent evidence suggests that the clone was misidentified. Instead it appears that a microsomal membrane protein, rather than lumenal PDI, is the monodeiodinase (25).

When PDI was first found to exhibit multiple activities in a single species, it became important to resolve the question of whether it was actually a single multifunctional protein or a family of highly homologous proteins, each possessing a different activity. The first evidence strongly suggesting a single protein was that Southern analyses of human genomic DNA using a probe constructed from the cDNA encoding the human β-subunit f prolyl hydroxylase, *i.e.* PDI, indicated the presence of a single gene (8). The absence of other cross-hybridizing genes indicated that the gene encoding for β-subunit of prolyl hydroxylase was not one of a family of genes encoding closely related proteins.[3] Subsequently, we found that PDI purified from a single source (rat liver) had both PDI activity and peptide binding function and that these could not be separated by a variety of chromatographic procedures. Further evidence indicating identity of PDI and THBP was obtained by transforming either *Escherichia coli* or 3T3 fibroblasts with human THBP cDNA and determining that the transformed cells now expressed both THBP and peptide binding activity (26). These results indicated that indeed human THBP also possessed the peptide binding activity of PDI.

A Variety of Proteins Have Thioredoxin-like Active Sites

Thioredoxin is a ubiquitous polypeptide of approximately 100 amino acid residues found in both prokaryotes and eukaryotes. It is involved in a variety of redox reactions via its active site, which consists of two Cys residues in the sequence, -Trp-Cys-Gly-Pro-Cys-Lys-, that can reversibly form an intramolecular disulfide bridge. In PDI, two such thioredoxin-like sequences are found, both of which contain His in place of Pro. A diagram of the structure of PDI showing these thioredoxin-like sequences, as well as the location of two other Cys residues in the protein, is shown in Fig. 1. Interestingly, very recently a 21-kDa periplasmic enzyme required for disulfide bond formation in *E. coli* containing a single thi redoxin-like active site, but with no other significant sequence similarity to thioredoxin or PDI, has been identified (27). Unlike PDI, this protein appears to be involved in the f rmation rather than the isomerization of disulfide bridges.

Murine ERp72, another protein resident in the lumen f the ER, contains three of the thi redoxin-like sequences. ERp72 contains extensive sequence similarities in the regions containing the PDI active sites, has a putative retention sequence (-KEEL), and is abundant in the ER (28). N physiol gical function has yet been attributed to this protein and, despite the presence of PDI active sites, it has not as yet been determined if ERp72 has disulfide isomerase activity. Recently it was reported that human deoxycytidine kinase contained three thioredoxin-like active sites and a sequence almost identical to that of the murine ERp72 cDNA (29). However, subsequently it was demonstrated that the so-called "kinase" cDNA encodes the human homolog of ERp72 rather than deoxycytidine kinase (30).

Two thioredoxin-like sequences also appear in another 57-kDa enzyme, phosphatidylinositol-specific phospholipase C isoenzyme I (31), although it is troubling that this isoenzyme does not display sequence homology to other sequenced PI-specific phospholipase Cs.

Recently it has been reported that the β-subunits of the gonadotropic hormones follitropin and lutropin also contain sequences resembling those of the thioredoxin active sites (32). In these glycoprotein hormones, which are dimers, the α-subunit is highly conserved, whereas the β-subunit defines the individuality of the various hormones. However, both hormones were shown to function very effectively in renaturation of RNase and, in fact, were more active than thioredoxin. An interesting hypothesis (32) is that upon binding to their receptors these proteins, via the action of their thioredoxin-like active sites, might catalyze a conformational change in the receptor that could elicit the signal transduction process. In this way these hormones would act as disulfide isomerases in a highly substrate-specific fashion.

Studies on the Mechanism of Isomerase Activity

Chemical modification of the Cys residues in PDI completely inhibits isomerase activity but only if the disulfide bridges have been reduced prior to alkylation (33). Although all six sulfhydryls (see Fig. 1) can be alkylated in reduced, denatured PDI, Hawkins and Freedman (33) found alkylation of only two of the sulfhydryls with pK values of 6.7 led to inactivation of the molecule. Their data suggest that two Cys residues, perhaps one in each of the cysteine pairs in the -Cys-Gly-His-Cys- sequences, have a low pK, indicating greater reactivity at physiologic pH. Inspection of the active site sequences indicates that in every case one of the Cys residues is adjacent to a basic amino acid residue; perhaps their cationic side chains stabilize the sulfhydryl in the unprotonated thiolate form.

The study of peptides as inhibitors of PDI activity discussed earlier has yielded a possible kinetic mechanism for the disulfide isomerase reaction (20) involving an ordered addition of substrates (34) and the formation of a ternary complex, rather than a double displacement or ping-pong mechanism. In this respect it is of interest that recently a covalent intermediate between PDI and ribonuclease has been detected during the regeneration of native ribonuclease (35).

Site-directed mutation of either one of the thioredoxin-like active site sequences caused loss of 50% of PDI activity, whereas mutation of both caused 100% loss of activity (36). Although this result suggests that each thioredoxin-like active site is independently active, recently Hawkins *et al.* (37) have demonstrated cooperativity between PDI active sites. In order to distinguish between active site on interactions within the monomer or in the homodimer, Hawkins *et al.* (37) examined the spatial relationships between the two active site sulfhydryls by chemical cross-linking using derivatives of the homobifunctional cross-linker, bisiodoacetamide. They were able to determine the cross-linked sulfhydryls to be located within different -Cys-Gly-His-Cys- sites of the same PDI molecule.

Although thioredoxin has a high degree of sequence similarity to PDI in the active site regions (Table I) and thioredoxin expresses some PDI activity, this protein is not as efficient as PDI in catalyzing the refolding of improperly f lded RNase (38). As noted above *E. coli* thioredoxin contains a Pro instead of a

[3] R. Noiva and W. J. Lennarz, manuscript in preparation.

His at residue 34 between the active site Cys residues. Krause *et al.* (39) have carried ut site-directed mutagenesis to convert this Pro to His in *E. coli* thi redoxin. The mutagenized thi redoxin had an increased redox potential, resulting in a significant increase in its ability to serve as a substrate for thioredoxin reductase and a decrease in its ability to reduce protein disulfides. It will be of interest to determine if the mutagenized thi redoxin catalyzes the f lding f reduced, denatured RNase, as it should behave more similar to PDI than to wild type thioredoxin.

The issue of the catalytic nature of the disulfide isomerase reaction, and whether PDI is a *bona fide* enzyme, has been raised because of its apparent low activity (40). Creighton *et al.* (41) followed the effects of PDI on the refolding of bovine pancreatic trypsin inhibitor and RNase. They determined that PDI was a true catalyst in several respects. First, PDI catalyzed the isomerization of many molecules of substrate per molecule of PDI. Second, PDI did not change the intermediates in the folding process but did alter the kinetics of their appearance. Finally, it was noted that PDI increased the kinetics of steps involving both disulfide bond reformation and major conformational changes in the polypeptide chains. Thus, PDI does not alter the pathway of folding but simply increases the rate of the overall process, indicating PDI does function as an enzyme.

Little is known about the redox environment of the lumen of the ER or even the identity of the major redox pair that presumably buffers this compartment. Experiments by Lyles and Gilbert (42) have demonstrated the importance of the redox state of the entire system in the disulfide isomerase reaction. Changes in the concentration of GSH and GSSG resulted in changes in both the PDI-catalyzed and uncatalyzed refolding of reduced, denatured RNase. The ratio of the velocity of the reaction catalyzed by PDI to the uncatalyzed reaction reached a constant when $[GSH]^2/[GSSG]$ was >1 mM, indicating that the reduced, dithiol form of PDI is required for catalytic activity. As noted by Lyles and Gilbert (42), their kinetic studies indicating a requirement of a dithiol form of PDI for activity are in agreement with chemical modification studies (33). Thus, it is obvious that the redox conditions are important for PDI activity, and it will be of interest to compare the redox conditions in the ER with the optimal redox requirements for PDI activity determined with the isolated enzyme.

Lyles and Gilbert (43) have also examined whether PDI itself, in the absence of any redox pair, might be acting as a redox reagent, given that its concentration in the lumen of the ER may be equivalent to that of the newly synthesized proteins undergoing folding. Their results confirmed that, in the absence of a redox buffer, PDI in stoichiometric quantities is sufficient to bring about refolding of RNase. Interestingly, PDI has been demonstrated to be able to substitute for thioredoxin in NADPH-dependent disulfide reduction reactions (44). PDI was found to be a substrate for calf NADPH-dependent thioredoxin reductase with a similar K_m to *E. coli* thioredoxin, and was able to reduce protein disulfides. Although Lundstrom and Holmgren (44) were able to demonstrate that thioredoxin is a stronger reductant than PDI, the presence of PDI in a concentration above the K_m and its ability to utilize reducing equivalents from the NADPH-dependent thioredoxin reductase to reduce protein disulfides suggest this system may have some physiological importance. The above results suggest that PDI could serve a mixed role as both an enzyme and a donor of redox equivalents.

Are There Multiple Binding Sites in PDI?

Given the apparent multifunctionality of PDI, the question of a single *versus* multiple binding sites is relevant. One possibility is that because the diversity f functions ascribed to PDI involves interactions with proteins or peptides there might be a single binding site on PDI that recognizes the polypeptide backbone as it is translated and translocated, and that th catalytic residues for the enzymatic functions could exist at separate sites. The active site of prolyl hydroxylase has been identified using affinity labeling with a synthetic peptide substrate (45). The results indicate that the pro-α procollagen peptide binding site resides on the α-subunit f the enzyme rather than on the β-subunit (PDI).

We have attempted to determine whether the peptide binding to PDI (17) occurs at the active site of disulfide isomerase. To investigate the relati nship f the peptide binding property and the disulfide isomerase activity of PDI, we modified the Cys residues using the sulfhydryl-specific reagents iodoacetic acid or *N*-ethylmaleimide.[3] Although such treatment completely inhibited the PDI activity, no inhibitory effect on peptide binding was noted. These results demonstrate that the active site Cys residues are not directly involved in peptide binding.

Tsibris *et al.* (46) have noted a region in PDI with similarity to the estrogen receptor, namely PDI residues 120–163 and 182–230 corresponding to estrogen receptor residues 350–392 and 304–349 (see Fig. 1). These regions are both contained within the steroid binding domain of the receptor (residues 301–552 (47)), suggesting that PDI might have similar steroid binding properties. In fact, estrogen causes specific noncompetitive inhibition of PDI activity and blocks the chemical cross-linking of PDI to its substrate (46). However, the observed level of inhibition was very low when performed using purified PDI preparations. Clearly, at present, the physiological function of the putative estrogen binding site and the relationship of this site to the other active sites of PDI is unknown.

What Is the Biological Function of PDI?

It is clear that PDI is a required subunit of the prolyl hydroxylases thus far described except for that in *Chlamydomonas.* However, this does not explain why PDI is so abundant in the lumen of the ER and occurs in vast excess of the α-subunit. Although it seems clear that proteins contain all the information required for proper folding in the absence of enzyme, PDI may be essential for this process to proceed at a physiologically relevant rate. Evidence that PDI does, in fact, catalyze protein folding within the ER comes from studies by Bulleid and Freedman (48). In their study, dog pancreas microsomes were depleted of their lumenal contents, including PDI, and then tested for their ability to synthesize mature, native γ-gliadin, a plant storage protein. These lumenally depleted microsomes were functional in translating γ-gliadin and translocating it into the lumen but were unable to properly fold it into its final, stable form. Addition of oxidizing equivalents alone to the lumenally depleted microsomes in the form of GSSG did not restore folding capability, but repletion with PDI did. In light of the recent studies by Lyles and Gilbert (42, 43) discussed above, it would seem important to repeat these repletion experiments altering the redox composition of the buffer to ensure that conditions were optimal for nonenzymatic disulfide bond formation.

Recent studies on PDI in *Saccharomyces cerevisiae* may provide further information concerning the physiological function of PDI. PDI isolated from yeast has a M_r of 70,000, rather than the 57,000 noted for other organisms (49). The yeast PDI cDNA was cloned, sequenced, and shown to have sequence similarity with rat PDI (50). Subsequently, Tachikawa *et al.* (51) cloned and sequenced the yeast PDI genomic DNA. In addition, LaMantia *et al.* (50) were able to demonstrate that yeast PDI, like rat PDI, binds photoreactive peptides. Several mutants containing gene disruption/deletion mutations in PDI were prepared and partially characterized (50). A mutant containing a deletion of most of the coding region of PDI was nonviable, indicating PDI plays an essential role in folding of newly synthesized proteins. Interestingly, removal of 36 or 174 amino acids from the COOH terminus resulted in viable yeast mutants whose only phenotype was a slower rate of germination. Site-directed mutations of the yeast PDI gene should be extremely useful in studying the mechanism of PDI activity and its biological function.

Subcellular Distribution of PDI

PDI is a soluble protein located in the lumen of the ER (52) and is free of any stable interaction with the microsomal membrane (53). PDI retention in the ER is mediated through the peptide sequence -KDEL (-HDEL in yeast) at the COOH terminus of PDI (see Fig. 1) (54). The mechanism of retention involves

recycling of PDI back to the ER from a compartment located between the ER and the Golgi (for review see Pelham (55)). A candidate for the -KDEL (-HDEL) receptor, the product of the *ERD* 2 gene, has been identified in *Kluyveromyces lactis* and *S. cerevisiae* (56, 57). The nature of the interaction with this receptor and its role in the recycling process remain to be established. However, our data indicating PDI is freely soluble upon mechanical disruption of microsomes (53) are consistent with the idea of sorting of lumenal proteins from secretory proteins and return from some salvage compartment, rather than retention in the ER by binding to an ER-associated receptor. It was recently noted that in tissues that are highly secretory, such as the pancreas, PDI containing the -KDEL sequence can be found in secretory vesicles, indicating that it has escaped the -KDEL retrieval mechanism (58). Similar observations have been made in another highly secretory organ, hen oviduct.[4] However, this generalization about "overloading" may be an oversimplification, since Dorner *et al.* (59) showed that, although overproduction of ERp72 or PDI leads to its secretion, other proteins containing recognition signals were not secreted. Clearly, this result cannot be explained by postulating a common, saturable retention mechanism for all lumenal proteins.

The cell's ability to recycle PDI to the ER and the apparent peptide binding activity of PDI suggests that PDI may associate with proteins on two levels: with native proteins as subunits of enzymatic complexes and with recently synthesized proteins during their processing in the ER. In both cases the ability of PDI to recycle to the ER could be functionally important. On one hand, proteins that do not contain the -KDEL retention sequence but are known to catalyze enzymatic reactions in the ER may be retained and may increase their residence time within the ER by virtue of their binding to PDI. On the other hand, proteins that are newly synthesized and have not yet assumed their native state may, by binding to PDI, continue to recycle back to the ER until they have been completely processed. The presence of PDI at levels equivalent to, or greater than, that of proteins being synthesized in the ER and the inducibility of PDI synthesis in response to many factors which induce secretory protein synthesis in general would also support this hypothesis.

Perspective

While all of the above results provide new insights into the properties of PDI, the fact is that its precise function remains unknown. Clearly it can reduce "incorrect" disulfide bonds, as in the case of scrambled oxidized RNase, so that ultimately the "correct" disulfide bonds in this protein can form. But this obviously is a unique *in vitro* situation, and the real issue is whether it functions to undo incorrectly formed disulfide bonds *in vivo*. As noted above, experiments indicate that in the absence of PDI the native conformation of a plant protein is not achieved. But this finding does not provide direct evidence that PDI functions to remove incorrect disulfide bonds by isomerization. Indeed, recent work on bovine pancreatic trypsin inhibitor indicates that incorrect disulfide bonds are not present on any of the abundant folding intermediates (60). This observation, which contradicts pioneering studies by Creighton and co-workers (see Creighton and Goldenberg (61)), who found intermediates containing incorrect disulfide bonds during trypsin inhibitor folding, raises the question of the very existence of such non-native bonds *in vivo*. If, in fact, non-native disulfide bonds do not form *in vivo*, what does PDI do? Obviously, we do not yet know, but given the recent progress in studying this novel protein, it seems likely that a clearer picture of its biological function(s) will emerge.

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[4] G. L. Decker and W. J. Lennarz, unpublished observations.